USE OF EPR SPIN-TRAPPING TECHNIQUES TO DETECT RADICALS FROM RAT LUNG LAVAGE FLUID FOLLOWING SULFUR MUSTARD VAPOR EXPOSURE

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ABSTRACT

Although well known for skin vesicating properties, pulmonary damage and associated infections account for most of the mortality associated with sulfur mustard (HD). We have employed an *in vivo* HD vapor exposure model, bronchoalveolar lavage and histopathology in conjunction with electron paramagnetic resonance (EPR) techniques to provide evidence for HD-induced (free radical/lipid peroxidation associated) lung injury. Anesthetized rats were intratracheally intubated and exposed to 0.35 mg HD vapor over 50 min. Immediately, 1 hr or 24 hr after exposure, lungs were lavaged with the spin trap, alpha-phenyl-t-butyl nitrone (PBN; 0.35 mg/ml). Recovered lavage fluid was assayed by EPR spectroscopy for radical spin adducts. Airway lipid extracts were assayed for thiobarbituric acid reactive products (TBARs); while separate groups of rats were used to evaluate histopathology. EPR results show the presence of an ascorbyl radical at 1 and 24 hr, and a carbon centered PBN spin adduct at 24 hr, both indicative of lipid peroxidation. TBAR (A_{532nm}) formation was also detected at 24 hr. Histopathology revealed multifocal separation of the bronchial epithelium from the submucosa with little or no alveolar involvement at 24 hrs. These studies provide evidence that HD may affect lungs by a free radical mechanism which produces membrane and other tissue damage.

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INTRODUCTION

Sulfur mustard (HD) is well known for its vesicating effects on epithelial tissues. The use of this agent in WWI and the Iran/Iraq conflict are well documented, and the threat of HD use was a concern in the Gulf War. Papirmeister et al. (1985) proposed a hypothesis for sulfur mustard vesication which suggests HD cross-links DNA strands leading to depurination, DNA strand breaks, and the depletion of cellular NAD followed by cell death with vesication localized at the site of exposure. In a recent report, Elsayed et al. (1992) indicated that subcutaneous exposure to a monofunctional HD analog caused pulmonary and other systemic effects removed from the site of exposure; such effects are thought to be mediated by free radicals. Indications of oxidative stress (i.e., lipid peroxidation and changes in antioxidant enzyme activities) in conjunction with depletion of glutathione was used by these authors as indirect evidence for the involvement of reactive oxygen species.

Historically, pulmonary damage and associated secondary infections have accounted for most HD-induced mortality (Papirmeister et al., 1991). We are examining the possibility that HD damage may also be associated with the production of free radicals and reactive oxygen species. Free radicals are, in general, very short-lived under physiological conditions, so direct detection is difficult in a biological system. One way to overcome this limitation is through the use of electron paramagnetic spin trapping techniques. Electron paramagnetic spin traps react with free radicals to form longer lived radicals/spin adducts. Structural differences between spin traps allows trapping of specific types of free radicals and can also help to identify the origin of the radical. Resulting electron paramagnetic resonance spectra and their corresponding coupling constants are like fingerprints and serve to identify particular radicals.

Bronchoalveolar lavage is a technique which can be extremely useful in the biochemical assessment of pulmonary health status (Henderson, 1989). Lungs can be lavaged tracheally with saline, or some other lavage fluid, which is then removed by low pressure vacuum. The recovered lavage fluid can then be assayed for a variety of biochemical markers of pulmonary disturbance.

The thiobarbituric acid (TBA) reaction is widely used in the detection of lipid peroxidation. The assay is based on the TBA catalyzed decomposition of lipid peroxide (e.g., L-OOH) and ultimate formation of malondialdehyde and other TBA reactive products (TBARs).

In this work, we have employed an in vivo HD inhalation exposure model, bronchoalveolar lavage in conjunction with electron spin trapping techniques, the TBA assay, and histopathology to provide evidence for HD-induced lung injury, lipid peroxidation and associated free radical production.

METHODS

SULFUR MUSTARD INHALATION EXPOSURE

Anesthetized rats were intubated with a modified Pasteur pipette to a point in the trachea between the larynx and the bronchi. Sulfur mustard (0.35 mg) in EtOH (100 µl), or EtOH alone (control), was placed in a water jacketed (37° C) vapor generator and the rats were exposed for 50 min, by which time the sample was completely removed. This passive exposure system involved a check

valve at each end to insure that the rats' only source of air during the exposure was through the vapor generator (Fig. 1). Exhaled air passed through a charcoal-filtered bleach trap to remove any remaining HD.

BRONCHOALVEOLAR LAVAGE

At 0, 1 or 24 hrs post-exposure, rats were re-anesthetized if necessary and exsanguinated through the abdomen. The trachea was isolated, nicked, and a 16 ga gavage needle was inserted and secured with suture. The lungs were lavaged with 1 ml of the spin trap [alpha-phenyl-t-butyl nitrone (PBN); 0.35 mg/ml]. The recovered lavage fluid was scanned by EPR spectroscopy.

THIOBARBITURIC ACID (TBA) ASSAY

The trachea and as much upper airway as possible were separated from the lungs and homogenized for lipid extraction. These airway lipid extracts were assayed for thiobarbituric acid reactive products (TBARs). Test material was heated with TBA under acidic conditions and the formation of a pink chromogen was measured spectrophotometrically at 532 nm. The TBA assay was only run on rats lavaged 24 hrs post exposure.

HISTOPATHOLOGY

Groups of six HD-exposed rats were used to evaluate histopathology by light microscopy at 0, 1, 4, 6, 12, 18 and 24 hr post exposure. Rats were re-anesthetized, if necessary, at the appropriate time point, exsanguinated, and the lungs removed for intratracheal fixation with 4CF-1G for 24 hrs at 30 cm fluid pressure.

RESULTS

Results from studies using the spin trap PBN show that, when rat lungs are lavaged immediately after the exposure (Fig. 2), the control and exposed animals give a comparable scan with negligible signals. At 1 hr (Fig. 3, B), EPR spectra indicated the presence of an ascorbyl radical (first shown by Yamazaki et al., 1960) with a hyperfine coupling constant a_H = 0.187 mT. The ascorbyl peaks are due to the detection of the actual radical, not formation of a spin adduct. At 24 hr, there is an ascorbyl peak, and indications of a poorly defined PBN adduct in the control (Fig. 4, A) which occurred occasionally and was probably due to the physical insult of the intubation and lavage. In the HD-exposed rat (Fig. 4, B) the ascorbyl radical peaks and the carbon centered PBN spin adduct (a_N= 1.65 and a_H= 0.35 mT) are present, both greater in amplitude than the control, and formed in response to lipid peroxidation. To verify this, the in vitro peroxidation reaction was performed (Fig. 4, C) and it generated a PBN spin adduct identical to the carbon centered radical. Again, both radicals detected by EPR, the ascorbyl and the carbon centered spin adduct, indicate that lipid peroxidation is occurring in the rat lung, primarily in response to HD vapor.

TBA ASSAY

The TBA assay was run as an additional check for lipid peroxidation. In this assay, a significant ($p<0.05$) increase in the formation of TBARs in rats lavaged 24 hrs after HD exposure, compared to EtOH exposure was detected.

PATHOLOGY

Observations of gross pathology in HD-exposed rats include multifocal hemorrhages on the lung surface beginning at 6 hrs, and atelectasis and edema of the post-caval lobe beginning at 12 hrs.

Histologically, the EtOH controls exhibited no change in pathology. Results at 0 and 1 hr showed little or no HD effect. Lesions in HD-exposed rats were primarily confined to conducting airways (trachea, bronchi and larger bronchioles). Isolated tracheal and bronchial epithelial necrosis at 4 hrs (evidenced by pyknotic nuclei at the junction of the epithelium and submucosa), and epithelial sloughing (6-12 hrs) was followed by the formation of pseudomembranes within the airways (Fig. 5). In almost all cases there was a progressive depletion of the bronchiolar associated lymphoid tissue (BALT), with necrosis of the lymphoid cells at 12 hrs. Pseudomembranes adhered almost exclusively to de-epithelialized areas overlying the BALT. Peribronchiolar and perivascular edema were present at 24 hrs (Fig. 6). Small bronchioles and alveoli appeared relatively unaffected and few inflammatory cells were observed at any time point.

DISCUSSION

The EPR data demonstrate the presence of an ascorbyl radical (1 hr) and a carbon-centered radical (24 hr) which both indicate that lipid peroxidation has occurred in response to HD vapor exposure. These radicals may be used as an indicator of HD-induced membrane damage. Observation of the ascorbyl radical preceeded alterations in pathology. Further evidence of lipid peroxidation was provided by the significant increase in the production of TBARs at 24 hr. In this study, although evidence of peroxidation of membrane lipid (ascorbyl radical) was seen at 1 hr post-exposure, earlier detection is desirable to characterize initial radical formation. One possible way of doing this may be the technique of Kennedy *et al*. (1992) which involves administering the spin trap systemically prior to exposure. This might prove to be a more sensitive technique for detecting radical formation at earlier time points.

Early HD-induced pathology was mainly isolated to the conducting airways in this exposure model. The prominent changes in pathology included early selective epithelial and cartilage necrosis, progressive loss of BALT and overlying mucosa, followed by psuedomembrane formation. The bronchial epithelial separation is similar to the microvesicle formation (epidermal/dermal separation) reported for hairless guinea pig skin after HD (Mershon *et al*., 1990), which may suggest similar mechanisms of HD damage.

Respiratory effects are responsible for many of the clinical illnesses/mortality resulting from HD exposure. Therefore, identification of biochemical parameters which provide the earliest indications of pulmonary injury may contribute to an understanding of lung injury and the subsequent development of a potential antidote.

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Fig. 1

IN VIVO RAT PREPARATION

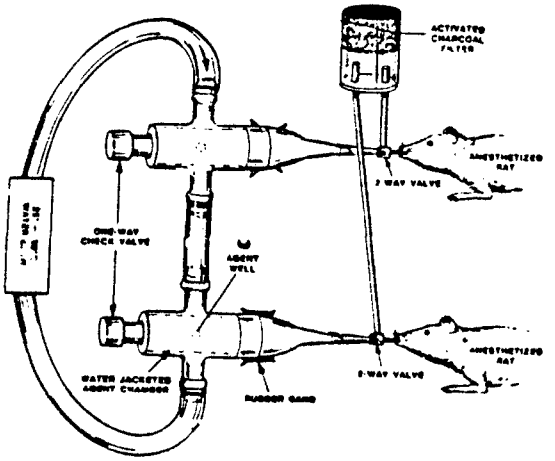


FIG. 2. EPR SPECTRA OF LAVAGE FLUID SAMPLES -- LAVAGED IMMEDIATELY POST-EXPOSURE (50 MIN) TO 0.35 MG VAPORIZED HD.

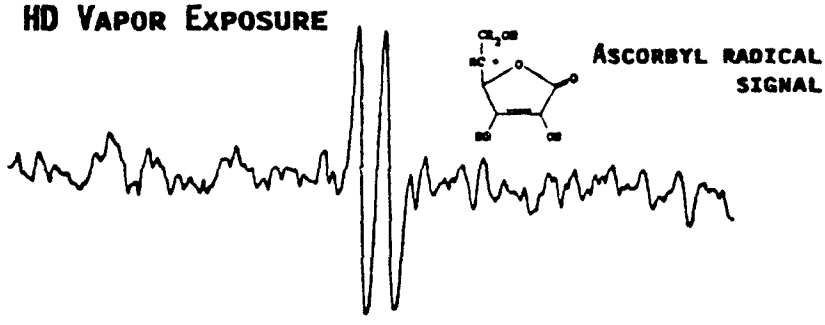
A) CONTROL

B) HD VAPOR EXPOSURE

FIG. 3. EPR SPECTRA OF RECOVERED BAL FLUID SAMPLES -- LAVAGED 1 HR POST-EXPOSURE (50 MIN) TO 0.35 MG VAPORIZED HD.

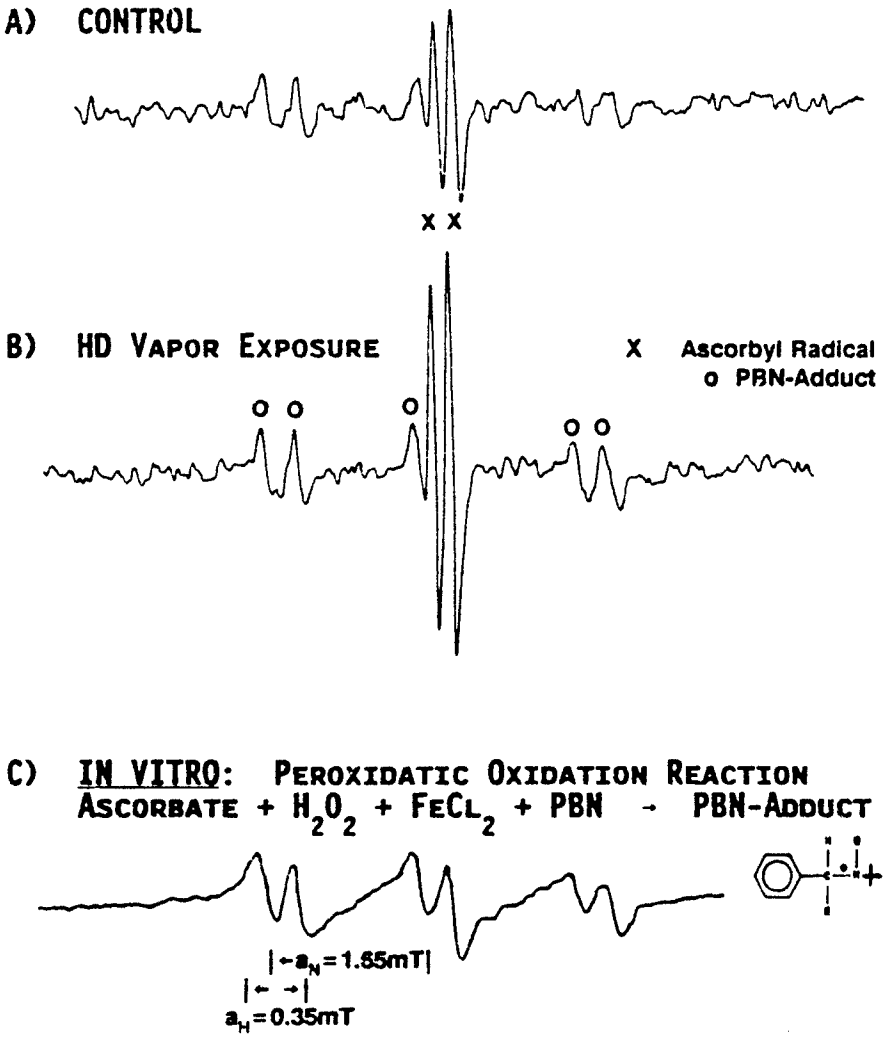
A) CONTROL

B) HD VAPOR EXPOSURE



A_N=0.187mT

FIG. 4. EPR SPECTRA OF RECOVERED BAL FLUID SAMPLES -- LAVAGED 24 HR POST-EXPOSURE (50 MIN) TO 0.35 MG VAPORIZED HD.



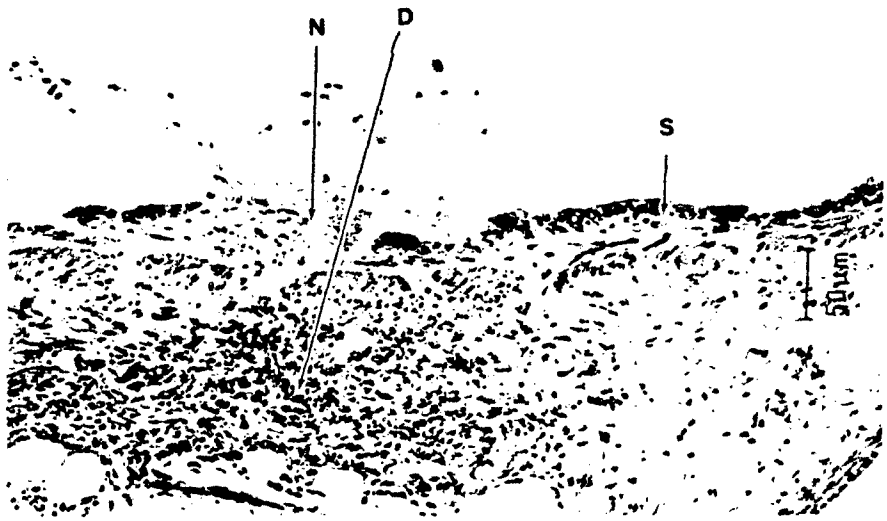


Fig. 5. Bronchiole (12 hrs): Necrosis and depletion (D) of the lymphoid tissue in the BALT. Epithelial necrosis (N) and loss with intraluminal exudate formation and adherence to de-epithelialized regions. Note necrosis and separation (S) at the mucosal-submucosal region (20X).

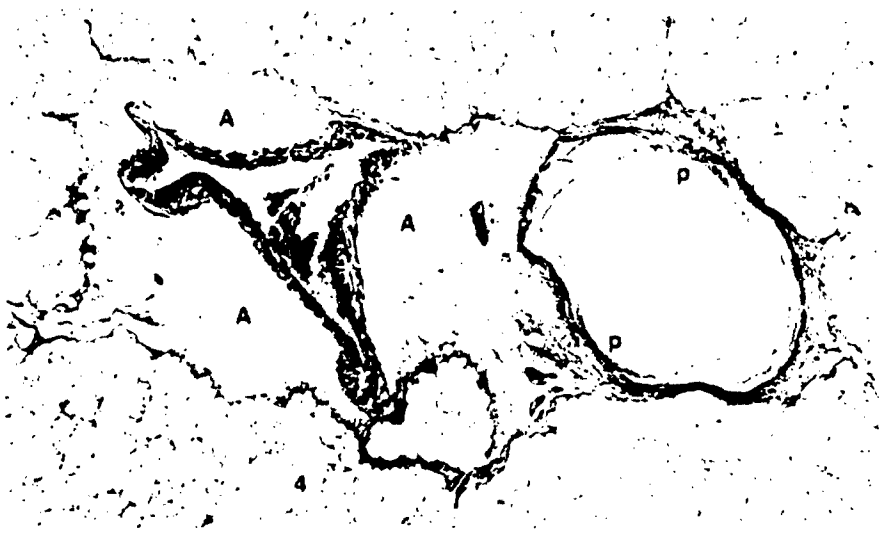


Fig. 6. Bronchiole and associated blood vessel (24 hrs): Marked peribronchiolar and perivascular edema (A). Intraluminal exudate formation with adherence to de-epithelialized regions overlying the BALT (P) (4X).